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(71) Applicant: CENTOCOR, INC. [US/US]; 244 Great Valley Parkway, Malvern, PA 19355 (US).

(72) Inventor: LoBUGLIO, Albert, F. ; 2204 Tanglewood Circle, Birmingham, AL 35294 (US).

(74) Agents: DeCONTI, Giulio, A., Jr. et al.; Hamilton, Brook, Smith & Reynolds, Two Militia Drive, Lexington, MA 02173 (US).

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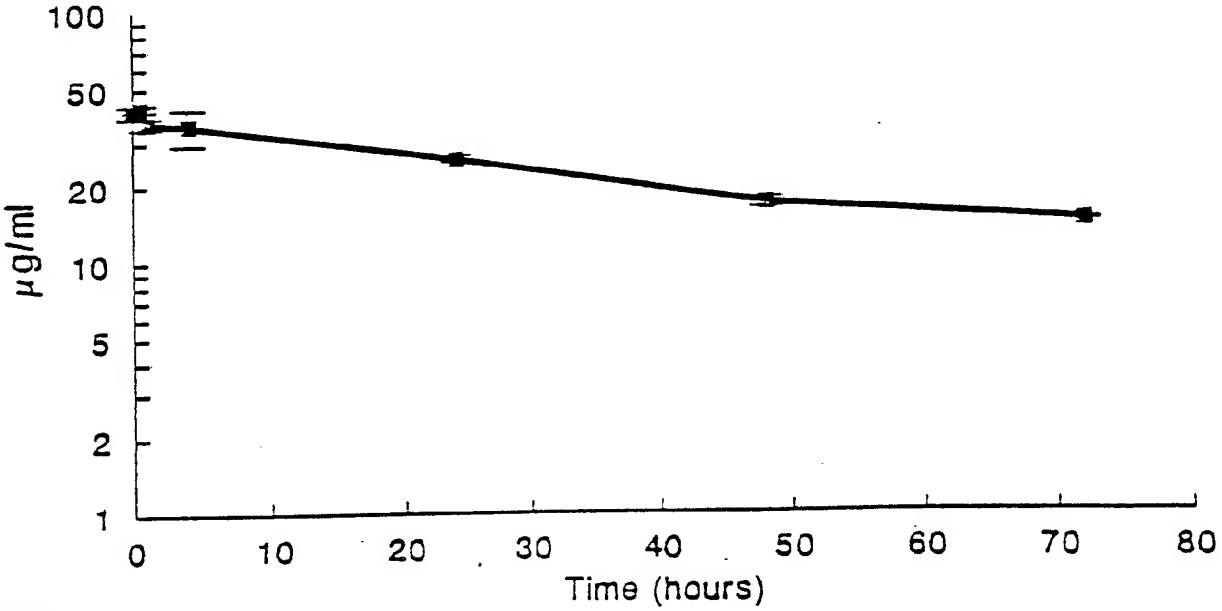

(57) Abstract

An immunometric assay is disclosed for preselected monoclonal antibody in a biological sample comprising, forming a complex of a first labeled anti-idiotypic monoclonal antibody, the preselected monoclonal antibody, and a second anti-idiotypic monoclonal antibody which can be bound to an insoluble substrate and detecting the amount of labeled antibody associated with the complex. The assay is characterized by employing first and second monoclonal antibodies which react with an idiotypic site on the preselected monoclonal antibody.

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ANTI-IDIOTOPIC IMMUNOMETRIC ASSAY

Background of the Invention

The recent development of monoclonal antibody technology has made it possible to produce unlimited amounts of homogeneous antibody preparations with single specificity. A variety of human monoclonal antibodies have been developed that are specific to antigens present in Rh blood groups [Evans, et al., J. Immunology, 140:941 (1988)], human immunodeficiency virus [Thompson et al., Immunology, 58:157 (1986)], malaria parasites [Udomsangpetch, et al., Science, 3:231 (1986)], endotoxin [Teng et al., PNAS, 80:7308 (1985)], human platelets [Nugent et al., Blood, 70:16 (1987)], and human tumor cells including melanoma [Yamaguchi et al., PNAS, 84:2416 (1987)], breast [Schlom et al., PNAS, 77:6841 (1980)] and colon cancer [Haspel et al., Cancer Research, 45:3951 (1985)]. Monoclonal antibodies such as these may be useful as therapeutic reagents. In addition, they can have both safety and product standardization advantages as compared to the use of human pooled hyperimmune sera. Furthermore, their use would have presumably fewer problems with immunogenicity than with use of murine monoclonal antibodies.

Clinical studies involving human monoclonal antibody products will require analysis of the pharmacokinetics of such reagents. These analyses will enable a rational design of treatment doses and administration schedules. Radiolabeled antibodies have been used to determine the pharmacokinetics of monoclonal antibody products in man [Sears, et al.,

J. Biol. Response Mod., 3:138 (1984) and Meeker, et al., Blood, 65:1349 (1985)], but this method has the disadvantage of requiring use of a radiolabeling procedure that might alter the molecule, thus resulting in alteration in pharmacokinetics. The pharmacokinetics of an injected dose can also be measured by quantitation of the circulating antibody at various times following administration [Khazaeli et al., Clin. Res., 35:615A (1988)]. This approach would require a specific and sensitive assay capable of detecting the monoclonal antibody in the presence of a vast excess of normal human immunoglobulins in serum.

Summary of the Invention

The present invention provides an immunometric assay for a preselected antibody in a biological fluid such as a blood sample. The assay comprises forming a complex of a first monoclonal antibody that is labeled, the preselected antibody, and a second monoclonal antibody and detecting the amount of label associated with the complex as indicative of the presence or the amount of preselected antibody in the biological fluid. The first and second monoclonal antibodies are specific for idiotopes of the preselected antibody. The preferred assay is a solid phase assay where at least one of the antibody constituents of the complex is bound to a solid phase, either before or after formation of the complex. The first antibody can be labeled before or after formation of the complex.

Brief Description of the Figures

Figure 1 shows a HPLC profile of purified anti-idiotypic murine monoclonal antibody, 15B2.2. (A) Analysis using a Bio-Rad Quick Check Analyzer with a Bio-Sil TSK 250 Column (300 x 7.5 mm). The sample was eluted with 10 mM phosphate, pH 6.8 containing 0.3 M NaCl, 10% dimethyl-sulfoxide (v/v) buffer at 1.0 ml/min. (B) Analysis of 15B2.2 post radiolabeling using HPLC as described above, with the addition of a radioisotope monitor to detect radioactivity.

Figure 2 shows cross reactivity of normal human immunoglobulins. Using a solid phase radiometric assay, increasing concentrations of HA-1A normal human IgG, IgM, IgA, IgE and IgD were incubated in the assay as described in materials and methods. (°) HA-1A, (o) other normal human immunoglobulins. The cross reactivity was calculated as less than 0.1%.

Figure 3 shows a dose response curve of HA-1A in solid phase radiometric assay. Increasing concentrations of HA-1A were incubated with 9B5.5-coated beads for 2 hours, washed and incubation was continued for 1 hour with ^{125}I-15B2.2. Bead associated radioactivity was determined and a standard curve was constructed using logit-log transformation regression analysis.

Figure 4 shows the serum concentrations of human IgM monoclonal antibody HA-1A in a patient who received 100 mg of the antibody. Serum samples obtained from the patient were assayed at appropriate dilution in the solid phase radiometric assay. The values are the mean of triplicate determinations ±1S.D.

Detailed Description of the Invention

The present invention provides a novel immunoassay for a preselected antibody in liquid sample. In the immunoassay of the invention, a complex is formed comprising a first labelled monoclonal antibody, the antibody to be measured (the "antigen"), and a second monoclonal antibody. The first and second monoclonal antibodies react with an idiotopic site on the antibody to be measured. Preferably, they are derived from the same hybridoma cell line and react with the same idiotopic site on the antigen. The immunoassay can be conducted in a reverse, simultaneous, or forward format. Following formation of the complex, the amount of preselected monoclonal antibody is quantified by detecting the amount of label associated with the complex. Preferred assays are those where the complex is immobilized on the solid phase.

This invention is based on the recognition that antibodies are themselves antigenic. It is possible, therefore, to induce antibodies that will recognize antigenic determinants on both the constant and the variable regions of immunoglobulin chains. Antigenic determinants on the variable regions of L and H chains that are associated with the antigen-binding site of an antibody are called idiotopes. The set of idiotopes on an individual antibody molecule defines the idiotype of that antibody.

The first and second monoclonal antibodies of this invention are anti-idiotypic. That is, they

will react solely with antigenic determinants (idiotopes) associated with the antigen-binding site of a pre-selected antibody molecule. Preferably, though not necessarily, these monoclonal antibodies are specific for a "private idiotope". The term "private idiotope" is based on the fact that several idiotopes may make up an immunoglobulin idiotype. Therefore, if two or more antibodies bind different antigens and are derived from independent V_H and V_L genes, each individual idiotope is considered "private" to one of the several antibodies. However, a portion of the V_H or V_L region away from the antigen binding site may have an idiotope in common between several antibodies. This particular antibody has a "public" idiotope. Since the "private" character of the antibodies of this invention may not be a necessary requirement, the more common anti-idiotypic "public" antibodies (Kiyotaki, M. et al., J. Immunol., 138:4150-4158 (1987)) can also be effective, although the likelihood of their cross-reacting with normal immunoglobulin would be higher than "private" anti-idiotypes.

Antibodies useful in the invention can be obtained by immunizing an animal, preferably a mouse, with a preselected antibody. Antibody produced cells are formed by fusing antibody producing cells from the immunized animal and an immortalizing cell such as myeloma.

Particularly preferred monoclonal anti-idiotypic antibodies of this invention are "private" anti-idiotypic antibodies, and are produced as

described herein. A particular example of such a monoclonal anti-idiotypic antibody is one that specifically reacts with idiotopes of human monoclonal antibody HA-1A.

In preferred embodiments, anti-idiotypic monoclonal antibodies of this invention are produced by hybridomas formed by fusion of: a) a mouse myeloma which does not secrete antibody with b) human lymph node spleen cells which secrete anti-idiotypic antibodies obtained from mice immunized against a preselected human monoclonal antibody (e.g., HA-1A).

The mice are immunized with a primary injection of monoclonal antibody followed by a number of boosting injections of the same antibody. During or after the immunization procedure, sera of the mice may be screened to identify those mice in which a substantial immune response to the antibody has been evoked. From selected mice, the spleen cells are obtained and fusions are performed. Suitable fusion techniques are the Sendai virus technique. Kohler, G. and Milstein, C., Nature 256:495 (1975), or the polyethylene glycol method, Kennet, R.H. in "Monoclonal Antibodies, Hybridomas -- A New Dimension in Biological Analyses", ed. R.H. Kennet, T. J. McKearn and K.B. Bechtol, Plenum Press, N.Y., 1980. Also, electrofusing techniques may be employed. Zimmerman, U. and Vienken, J., J. Membrane Bio. 67:165 (1982).

The hybridomas are then screened for production of anti-idiotypic antibody. A suitable preliminary screening technique is an enzyme-linked immuno-

sorbent assay (ELISA) using plates precoated with the monoclonal antibody of interest and control immunoglobulins. That is, solid phase immunoadsorbent is prepared by coupling, for example HA-1A, to an insoluble matrix. The immunoadsorbent is brought into contact with culture supernatants of hybridomas. Hybridomas secreting antibodies reactive with the preselected monoclonal antibody but not with other immunoglobulins are selected and subcloned. Anti-idiotypic antibodies can then be tested for "public" and "private" specificity using immunofluorescence analysis. Kiyotaki, M. et al., J. Immunol. 138:4150 (1987).

Monoclonal anti-idiotypic antibodies for use in the assays can be produced in large quantities by injecting anti-idiotype-producing hybridoma cells into the peritoneal cavity of mice and, after an appropriate time, harvesting ascites fluid from the mice which yields a very high titer of homogenous anti-idiotypic antibody. The monoclonal antibodies are isolated therefrom. Alternatively, the antibodies can be produced by culturing anti-idiotype producing cells in vitro and isolating secreted monoclonal anti-idiotype antibodies from the cell culture medium directly.

Because the anti-idiotypic antibodies of this invention discriminate between the preselected antibody and the normal suite of human immunoglobulins, they permit a sensitive immunochemical assay of the preselected monoclonal antibody. A particularly preferred type of immunochemical assay is a sandwich immunometric

assay in which antigen (i.e., natural or recombinant the monoclonal antibody) is measured directly by reacting it with murine anti-idiotypic antibody that is labeled, or capable of being labeled.

In sandwich assays of this invention, a complex is formed comprising: 1) a first antibody specific for an idiotope on a preselected monoclonal antibody, 2) the preselected antibody (the "antigen"), and 3) a second antibody specific for an idiotope of the preselected antibody. This first antibody is labeled either before or after formation of complex. The label can be attached directly or indirectly to antibody. For example, the antibody can be complexed with biotin, to which a label can be attached via avidin linkage.

The complex can be formed before it is immobilized onto a solid phase. In other embodiments, the complex can be immobilized on the solid phase at the same time that it is formed. In preferred assays, the antigen is immobilized on an immunoadsorbent which specifically "captures" or binds the preselected antibody ("antigen"). This immunoadsorbent is formed by affixing to it an antibody specific for an idiotope of the preselected monoclonal antibody. In preferred sandwich assays of this invention, two anti-idiotypic antibodies which recognize identical idiotopes on the antigen can be used. Thus, for most purposes, the same anti-idiotypic antibody used to form the immunoadsorbent can be used as the labeled antibody.

Sandwich assays may be performed in forward, reverse or simultaneous mode. In a forward sandwich assay for an antibody, a monoclonal antibody directed against an idiotope of the antibody is affixed to a solid phase. A liquid sample to be tested is incubated with the immunoadsorbent. Incubation is maintained for a sufficient period of time to allow the antibody in the liquid sample to bind to its immobilized anti-idiotypic antibody on the immunoadsorbent. After this first incubation, the solid phase immunoadsorbent is separated from the sample. The immunoadsorbent is washed to remove unbound monoclonal antibody and interfering substances, such as non-specific binding proteins, which may also be present in the liquid sample. The immunoadsorbent containing antibody bound to immobilized antibody is subsequently incubated with labeled anti-idiotypic antibody, specific for idiotope(s) on the monoclonal antibody. The incubation is carried out for a period of time and under conditions sufficient to ensure binding of the labeled anti-idiotypic antibody to the antibody. After the second incubation, another wash may be performed to remove unbound label from the solid phase immunoadsorbent. The labeled anti-idiotypic antibody bound to the solid phase immunoadsorbent is then measured, and the amount of label detected serves as a direct measure of the amount of antibody present in the liquid sample.

Anti-idiotypic monoclonal antibodies can provide the basis for an extremely sensitive forward

sandwich immunoassay for an antigen such as HA-1A, a human IgM monoclonal antibody to endotoxin. In one configuration, murine anti-idiotypic monoclonal antibody is used to form the immunoadsorbent and also serves as the labeled antibody. The assay is performed as outlined above. With these two antibodies, the assay is specific for HA-1A and highly sensitive. Levels of HA-1A in serum or tissue culture fluid at limiting concentrations of about 25 ng/ml can be detected.

The sandwich immunoassays may also be performed in reverse and simultaneous modes. In reverse modes, an incubation mixture is formed of the liquid sample to be tested and a soluble labeled anti-idiotypic antibody directed against an idiotope of a preselected antibody such as HA-1A. The mixture is incubated, then contacted with a solid phase immunoadsorbent containing an anti-idiotypic monoclonal antibody directed against the same or different idiotope of the preselected antibody. After another incubation, the immunoadsorbent is separated from the mixture and the label bound to the immunoadsorbent is taken as an indication of the amount of preselected monoclonal antibody in the liquid sample.

In the simultaneous mode, an incubation mixture is formed of the liquid sample containing monoclonal antibody to be measured (e.g., HA-1A), labeled anti-idiotypic antibody and the solid phase immunoadsorbent. After appropriate incubation, the solid phase immunoadsorbent is separated from the mixture

and the label associated with the immunoadsorbent is measured to give an indication of the amount of monoclonal antibody in the liquid sample.

For each incubation step in the various assay formats, the time and incubation conditions are selected to ensure optimal binding of antigen (i.e., monoclonal antibody) to the immobilized anti-idiotypic antibody and to labeled anti-idiotypic antibody. In the forward sandwich immunoassay, where two incubation steps are required, the solid phase immunoadsorbent containing immobilized anti-idiotypic antibody is incubated with the liquid sample for several hours at room temperature to obtain optimal binding. The parameters which yield optimal binding of monoclonal antibody reagent may be established for other formats of the immunoassay by no more than routine experimentation.

The immunoassays of this invention are used to detect and quantify monoclonal antibody in a liquid sample. Liquid samples include essentially all biological fluids such as blood, or blood-derived fluids such as plasma or serum, as well as urine, lymph, etc. Also, the liquid sample may be a sample of a liquid medium in which lymphocytes or other mammalian cells have been cultured. They may also be extracts or supernatants of microbial cultures.

The assays of this invention can be used to detect any monoclonal antibody capable of forming a suitable complex with anti-idiotypic monoclonal antibodies (i.e., labeled anti-idiotypic antibody preselected monoclonal antibody: unlabeled

anti-idiotypic antibody), mammalian, preferably human monoclonal antibodies. These monoclonal antibodies are generally useful as therapeutic reagents. Assay methods of this invention can be used to detect monoclonal antibodies raised against carcinoma cells, lymphoma cells, fibrin (e.g., T2G1, Kudryk, B. et al., Mol. Immunol. 21:89 (1984)), platelet (e.g., 7E3, European Patent Application No. 205,207) endotoxin (e.g., HA-1A) and many others. Monoclonal antibodies that are chimeric (i.e., those in which variable regions of antibodies from one mammal are joined to constant regions of antibodies from a different mammal), can also be detected in assays of this invention.

In selected solid phase immunometric assays of this invention, anti-idiotypic monoclonal antibodies reactive with preselected monoclonal antibodies can first be immobilized by affixing them to a solid phase to create an "immunoadsorbent". The anti-idiotypic antibody is therefore affixed to the solid phase before the three-part complex (i.e., labeled anti-idiotypic antibody: preselected monoclonal antibody: unlabeled anti-idiotypic antibody) is created. This tripartate or ternary complex can also be attached to a solid phase after the complex is formed. This can be accomplished, for example, by affixing avidin to the solid phase and allowing the ternary complex to form in solution, one anti-body of this complex being labeled with biotin.

Many types of solid-phases may be employed. Well-known solid phases include beads formed from

glass, polystyrene, polypropylene, dextran, and other materials; tubes formed from or coated with such materials, etc. The anti-idiotypic antibody can be either covalently or noncovalently bound to the solid-phase by techniques such as covalent bonding via an amide or ester linkage or adsorption. Those skilled in the art will know many other suitable solid-phases and methods for immobilizing antibodies thereon, or will be able to ascertain such using no more than routine experimentation.

In the various solid phase assays of this invention, the immunoadsorbent can be separated from incubation mixtures containing the liquid sample, the labeled antibody or both. Separation can be accomplished by any conventional separation, filtration, or centrifugation step. Preferably, the immunoadsorbent is washed prior to contacting it with a second incubation medium (e.g., a solution of labeled anti-idiotypic antibody and the preselected antibody) and prior to measuring the amount of label associated with the immunoadsorbent. The washing removes nonspecific interfering substances or excess label which may affect the accuracy and sensitivity of the assay.

In each of the immunoassays of this invention, monoclonal anti-idiotypic antibody directed against an idiotope of a preselected mammalian monoclonal antibody is also used as the labeled antibody (tracer). Such antibodies can be labeled directly with a radioactive material, such as ^{125}I; labeled with an optical label, such as fluorescent material; labeled with an enzyme; or labeled by some other technique. These antibodies can also be labeled

indirectly (i.e., by complexation with another labeled antibody).

To determine the amount of monoclonal antibody in the liquid sample, either the amount of label associated with the immunoadsorbent or the amount of unbound label, that is, labeled anti-idiotypic antibody which remains in soluble form, is measured. Generally, it is preferable to measure the label bound to the immunoadsorbent because at very low concentration of monoclonal in the sample, only small amounts of labeled anti-idiotypic antibody bind the immunoadsorbent. Thus, for accuracy the label associated with the immunoadsorbent should be measured directly. The label may be detected by a gamma counter, for example, if the label is a radioactive gamma emitter, of by a fluorimeter, if the label is a fluorescent material. In the case of an enzyme label, detection may be done by colorimetric methods employing a substrate for the enzyme.

The measured amount of label detected is then compared to a quantitative relationship between the amount of preselected label and the amount of monoclonal antibody. The quantitative relationship can be determined by performing the immunoassay with standards (i.e., liquid samples containing known amounts of monoclonal antibody). For several samples containing different amounts of preselected monoclonal antibody, the assay is conducted and the amount of label either bound or unbound to the immunoadsorbent is determined; a curve is con-

structed defining the quantitative relationship between the amount of label and the amount of monoclonal antibody. By reference to the curve, the amount of monoclonal antibody in a liquid sample containing an unknown amount of monoclonal antibody can be determined from the amount of label detected.

The immunoassays described provide rapid, highly sensitive, inexpensive and reproducible methods for detection and quantification of monoclonal antibodies. The assays provide a substitute for existing bioassays which are more time-consuming and variable and much less sensitive and specific. Thus, the assay reagents may be provided conveniently in kits.

The assays may be employed by hospitals or clinical laboratories to determine levels of therapeutic and/or diagnostic monoclonal antibodies in serum, plasma or other biological fluids of patients. The assay may also be used to monitor the amount of monoclonal antibody during the course of antibody therapy. This will be of predictive value in managing the course of treatment in a variety of disease states.

The reagents for performing the assays of this invention may be assembled in assay kits. For instance, a kit for performing an immunoassay for HA-1A would comprise a solid phase immunoadsorbent containing an anti-idiotypic antibody specific for one idiotope of HA-1A, a labeled anti-idiotypic antibody specific for the same idiotope of HA-1A and, optionally, an HA-1A standard.

The invention is further described in the following examples wherein all parts and percentages are by weight degrees are celsius.

Examples:

Materials and Methods

Gram negative lipopolysaccharide (LPS) was obtained from List Biological Laboratories, Inc. Campbell, CA. E. coli was purchased from Sigma Chemical Company, St. Louis, MO. Normal human IgG, IgM and Ig was supplied by Southern Biotechnology Associates, Inc., Birmingham, AL and normal human IgE and IgD was supplied by Behring Diagnostics, LaJolla, CA. ^{125}I was obtained from the DuPont Company, Wilmington, DE.

Production of HA-1A

The HA-1A monoclonal antibody was produced by a hybridoma cell line generated by fusion of splenic lymphocytes with a heteromyeloma cell line (Teng, N.N.A., K.S. Lam, F.C. Rieva and J.S. Kaplan, Proc. Natl. Acad. Sci. USA, 80:7308-7312 (1983) and Bron, D., M.B. Feinberg, N.N.H. Teng and H.S. Kaplan, Proc. Natl. Acad. Sci. USA, 81:3214 (1985)). The splenocytes were removed from a patient undergoing splenectomy during staging for Hodgkin's disease. The patient had been immunized with the J5 mutant of Escherichia Coli (E. coli) which expresses the core oligosaccharide common to the lipopolysaccharide (LPS) of all Gram-negative bacteria. The HA-1A monoclonal antibody cross-reacts with endotoxins

from a wide range of unrelated species of Gram-negative bacteria and protects against lethal Gram-negative bacteremia in mice (Teng, N.N.H., H.S. Kaplan, J.M. Herbert, C. Moore, H. Douglas, A. Wunderlich and A. Braude, Proc. Natl. Acad. Sci. USA, 82:1790 (1985)). HA-1A is a human IgM_k molecule. It was isolated and purified by Centocor (Malvern, PA) and provided as a solution of 5 mg/ml in 0.01 M Sodium phosphate, 0.3M NaCI, pH 7.2 buffer.

Production of Murine monoclonal anti-idiotypic antibodies to HA-1A

Lymph node cells from BALB/c mice immunized with HA-1A were fused with non-Ig producing cell line P3-X63-Ag8.653 (Kearney, J.F. et al., J. Immunol., 123:1548 (1979)). Culture supernatant from hybridoma containing wells were tested for antibody specificity using an enzyme-linked immunosorbent assay (Elisa) in 96-well plastic plates pre-coated with HA-1A or control immunoglobulins. Hybridomas that secreted antibodies reactive with HA-1A, but not with other immunoglobulins including IgM or IgA paraproteins and normal IgG were selected and subcloned by limiting dilution. The anti-idiotypic reagents were subsequently tested for "public" and "private" idiotypic specificity utilizing a two-color immunofluorescence analysis as previously described (Kiyotaki, M. et al., J. Immunol., 138:4150-4158 (1987)). The two cell lines having "private" anti-idiotypes (9B5.5 and 15B2.2) were injected intraperitoneally into pristane primed syngeneic mice for ascites production.

Purification of anti-idiotypic monoclonal antibodies

The ascites fluid was purified by passage over a Bakerbond ABx HPLC column (10x250 mm) equilibrated with 25mM MES buffer pH 5.5. After the elution of unbound proteins, a gradient from 0 to 100% of 1M NaOAc pH 7.0 was used over 60 min to elute the monoclonal antibody. The recovered antibody was analyzed for purity using a Quick-check analysis (BioRad, Richmond, CA). This analytic column was a Bio-Sil TSK-250 (7.5 x 300mm). The eluting buffer was .01M phosphate, 0.3 M NaCI, 10% dimethyl-sulfoxide (v/v) eluting at 1 ml/min.

HA-1A solid phase radiometric assay

Polystyrene beads, 6.4 mm diameter (Precision Plastic Ball, Inc., Chicago, IL), were coated with 200 µl anti-id 9B5.5 in phosphate buffered saline (PBS) at a concentration of 5 µg/ml. Beads were washed three times with PBS containing 2% bovine serum albumin (BSA) and .02% Tween 20 and allowed to stand in wash buffer for 1 hr at room temperature. The beads were air dried and stored at 4°C until used. Human monoclonal antibody HA-1A was diluted in normal human serum (NHS) at concentrations ranging from 12.5-6400 ng/ml. Standard, controls and patient serum at appropriate dilution were incubated (100 µl) for 2 hr in triplicate with coated beads on a laboratory rotator at room temperature. The beads were washed with 4 ml of PBS. One hundred µl of radiolabeled anti-idiotypic antibody 15B2.2 was added to each bead at a concentration of 1 µg/ml. The incubation was continued

for an additional 1 hr. The beads were washed again with 4 ml of PBS, transferred to clean tubes and the bead associated radioactivity was determined with a Micromedic Automatic Gamma Counter (Micromedic System, Inc., Horsham, PA) interfaced with an IBM System 2. A logit-data reduction program was used to generate the standard curve and the values of controls and patient samples.

Iodination of proteins

The purified monoclonal antibodies were labeled with ^{125}I by a modified method of Greenwood, et al., 1963. The radiolabeled monoclonal antibodies were analyzed using HPLC as described above, except that a radioisotope monitor was used to detect radioactivity in sequence with the U.V. monitor. The protein was measured in final preparation by the method of Lowry, O.H. et al., J. Biol. Chem., 193:265 (1951).

Example 1 Development of an Immunoassay for HA-1A

Murine Monoclonal Anti-idiotypic Antibody Preparation against HA-1A

Among 11 hybridomas producing antibodies which were reactive in an ELISA with HA-1A but not with other myeloma proteins or normal IgG, only two antibodies (9B5.5 and 15.B2.2) were found by two-color immunofluorescence analysis to be non-cross-reactive with plasma cells generated from pokeweed mitogen-stimulated blood lymphocyte (0.1%). The other 9 antibodies were cross-reactive

to the plasma cells on the order of 0.1-2.0%. Thus, antibodies 9B5.5 and 15B2.5 were designated as "private" anti-idiotypic antibodies. The monoclonal antibodies were purified as described above. The final products were concentrated to 5.0 mg/ml on an Amicon 8050 stirred cell filtration apparatus and were stored at 4°C. Figure 1 shows the HPLC profile of the purified monoclonal anti-id antibody 15B2.2 (A) and the radiolabeled 15B2.2 (B). There were no aggregates or break down products seen.

HA-1A solid phase radiometric assay

A number of studies were carried out to establish the standard conditions of this assay. Polystyrene beads were carried with varying amounts of 9B5.5 from 0.1 μg to 1.6 μg/bead. Maximum binding occurred at 1.0 μg/bead. The amount of radiolabeled 15B2.2 used in the assay was determined by incubating the 9B5.5 coated beads with HA-1A standards followed by incubation with varying amount of ^{125}I - 15B2.2. The 0.1 μg of ^{125}I - 15B2.2 was sufficient to have an excess of 15B2.2 available at all concentrations of standard. The two incubations were carried out at intervals varying from 30 min to 18 hrs at both room temperature and 37°C. Greater than 90% of maximum binding occurred with 2 hour incubation with sera and one hour incubation with radioactive 15B2.2. Binding was comparable at room temperature and 37°C. For convenience, room temperature incubations were used. Using the HA-1A assay as described above, increasing concentrations of HA-1A, normal human IgG, IgM, IgA, IgE and IgD .

were assayed (Fig. 2). The cross reactivity of these various immunoglobulin preparations was calculated as less than 0.1%. The sensitivity of the assay defined as 2 standard deviations above the nonspecific binding (normal human serum) was approximately 25 ng/ml. The linearity of the assay is best seen by logit-log analysis (Fig. 3). The assay is linear between 25 and 800 ng/ml.

Recovery and reproducibility studies were also carried out. Normal human serum was "seeded" with 0.07, 1.6, 8.2, 12.0 and 19.0 μg/ml of HA-1A and subsequently assayed at appropriate dilutions in three separate assays. As seen in Table 1, the average percent recovery was 116±4% and the range was 113-123% over this wide spectrum of serum concentrations.

Table 1: The recovery of HA-1A in human serum

HA-1A added ug/ml	HA-1A detected ug/ml ± S.D.	% Recovery
.07	.08 ± .01	114
1.6	1.97 ± .06	123
8.2	9.4 ± 0.4	115
12.0	13.6 ± 0.87	113
19.0	22.47 ± 1.52	118

The inter-assay variance was examined by assay of three concentrations of HA-1A (200, 1000 and 2500 ng/ml in human serum) as positive controls in 10 consecutive independent assays. The means for the

three HA-1A levels were 206 ± 12, 981 ± 65 and 2573 ± 161 ng/ml, respectively (a coefficient of variation of between 5.8 and 6.6 percent).

Since this antibody (HA-1A) is likely to be used in patients with gram negative sepsis, the effects of bacteria and LPS on the assay HA-1A in human serum was studied. Human sera containing 20 μg/ml HA-1A was incubated with varying numbers of bacteria (E. coli) or varying concentrations) LPS at 37° for 20 minutes and then assayed for HA-1A content (Table 2). These additives had no adverse effects in detection of HA-1A.

Table 2: Effect of E. coli and LPS on detection of HA-1A in human serum.

Serum + 20 μg/ml HA-1A plus:	Mean HA-1A measured μg/ml*
nothing	21.9 ± .49
<1 CFU E. coli/ml	21.4 ± 1.8
10 CFU E. coli/ml	20.2 ± 1.8
80 CFU E. coli/ml	21.02 ± 1.5
0.20 μg/LPS	22.95 ± 1.04
2.0 μg/LPS	20.5 ± 1.8
20.0 μg/ml LPS	19.9 ± 1.9

Example 2 Detection of HA-1A from human patient

A patient was given 100 mg. of HA-1A and serial serum samples drawn for quantitation of HA-1A. As seen in Figure 4, mean peak serum concentration after infusion was 36.6 μg/ml, which is 101.5% of

the predicted value, based on the patient's calculated plasma volume. The data fit a one compartment plasma disappearance model having a mean plasma half-life of 24.5 hrs (Sisson, 1983). This illustrates the ability of this assay to be used for pharmacokinetic studies.

Equivalents

Those skilled in the art will recognize or be able to ascertain, using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS

1. An immunometric assay for a preselected antibody in a biological fluid comprising:
   a. forming a ternary complex of:
      i. a first monoclonal antibody that is labeled, or capable of being labeled, specific for an idiotope of the preselected antibody;
      ii. a second monoclonal antibody specific for an idiotope of the preselected antibody; and
      iii. the preselected antibody;
   b. detecting the amount of label associated with the complex formed from the components in step (a), as an indication of preselected antibody in the fluid.

2. An assay of Claim 1, wherein the first labeled monoclonal antibody is labeled with Iodine-125.

3. An assay of Claim 1, wherein the second monoclonal antibody is immobilized on a solid phase before or after formation of the ternary complex.

4. An assay of Claim 1, wherein the first and second monoclonal antibodies are anti-idiotypic antibodies specific for the same private idiotope on the preselected monoclonal antibody.

5. An assay for a preselected antibody in a blood-derived sample, comprising the steps of:

a. forming an incubation mixture of the sample and a solid phase immunoadsorbent containing monoclonal anti-idiotypic antibody affixed to a solid phase, this antibody specific for an idiotope of the preselected antibody;

b. incubating the incubation mixture under conditions and for a period of time sufficient for preselected antibody in the liquid sample to bind to the immunoadsorbent;

c. thereafter separating the immunoadsorbent from the liquid sample;

d. forming an incubation mixture of the immunoadsorbent and soluble labeled monoclonal anti-idiotypic antibody, this antibody specific for an idiotope of the preselected antibody;

e. incubating the mixture under conditions and for a period of time sufficient for the labeled antibody to bind any preselected antibody;

f. separating the solid phase immunoadsorbent from unbound labeled anti-idiotypic antibody;

g. detecting the amount of label bound to the immunoadsorbent or the amount of unbound, label; and

h. relating the amount of bound label or unbound label detected to a quantitative relationship between the amount of label and the amount of preselected monoclonal antibody to determine the amount of preselected antibody in the sample.

6. An assay of Claim 5, wherein the soluble labeled anti-idiotypic monoclonal is labeled with materials selected from the group consisting of radionuclides, enzymes and fluorescent agents.

7. An immunoassay of Claim 5, wherein the anti-idiotypic monoclonal antibodies are anti-idiotypic antibodies specific for the same private idiotope on the preselected monoclonal antibody.

8. An assay of Claim 5, wherein the preselected antibody is antibody HA-1A.

9. An assay for a preselected antibody in a liquid sample, comprising the steps of:

a. forming an incubation mixture of the sample and a soluble, labeled anti-idiotypic monoclonal antibody that is specific for an idiotope on the preselected monoclonal antibody;

b. incubating the incubation mixture under conditions and for a period of time sufficient for preselected monoclonal antibody in the liquid sample to bind to the labeled, soluble monoclonal antibody;

c. contacting a solid phase immunoadsorbent containing anti-idiotypic monoclonal antibody affixed to a solid phase, this antibody specific for an idiotope on the preselected monoclonal antibody with the incubation mixture;

d. incubating the components of step (c) under conditions and for a period of time sufficient for preselected monoclonal antibody bound to the labeled, soluble anti-idiotypic monoclonal antibody to bind the immunoadsorbent;

e. separating the solid phase immunoadsorbent from the incubation mixture;

f. detecting the amount of label bound to the solid phase immunoadsorbent or the amount of unbound label; and

g. relating the amount of label detected to a quantitative relationship between the amount of label and the amount of preselected monoclonal antibody to determine the amount of preselected monoclonal antibody in the liquid sample.

10. An immunoassay of Claim 9, wherein the anti-idiotypic monoclonal antibodies are specific for the same private idiotope of the pre-selected antibody.

11. An immunoassay of Claim 9, wherein the pre-selected monoclonal antibody is HA-1A.

12. An immunoassay of Claim 9, wherein the soluble labeled anti-idiotypic monoclonal antibody is labeled with materials selected from the group consisting of radionuclides, enzymes and fluorescent agents.

13. An immunoassay for preselected monoclonal antibody in a liquid sample, comprising the steps of:

a. forming an incubation mixture of
   i. liquid sample;
   ii. a solid phase immunoadsorbent containing immobilized monoclonal antibody that is specific for an idiotope of the preselected monoclonal antibody; and
   iii. labeled soluble monoclonal antibody that is specific for an idiotope of the preselected monoclonal antibody;

b. incubating the mixture under conditions and for a period of time sufficient for the preselected monoclonal antibody in the liquid sample to complex with both the immobilized monoclonal antibody and the labeled, soluble, monoclonal antibody;

c. thereafter separating the solid phase immunoadsorbent from the incubation mixture;

d. detecting the amount of label bound to the solid phase immunoadsorbent or the amount of unbound label; and

e. relating the amount of label and the amount of preselected monoclonal antibody to determine the amount of preselected monoclonal antibody in the liquid sample.

14. An immunoassay of Claim 13, wherein the immobilized and soluble monoclonal antibodies are specific for the same private idiotope of the preselected monoclonal antibody.

15. An immunoassay of Claim 13, wherein the preselected monoclonal antibody is HA-1A.

16. A forward sandwich immunoradiometric assay for preselected monoclonal antibody in a liquid sample comprising the steps of:

a. forming an incubation mixture of the liquid sample and solid phase immunoadsorbent comprising polystyrene beads to which is affixed anti-idiotypic monoclonal antibody specific for an idiotope of the preselected monoclonal antibody;

b. incubating the mixture at room temperature;

c. thereafter separating the immunoadsorbent from the liquid sample;

d. forming an incubation mixture of the immunoadsorbent and soluble ^{125}I-labeled anti-idiotypic monoclonal antibody specific for an idiotope of the preselected monoclonal antibody;

e. incubating the mixture at room temperature;

f. separating the immunoadsorbent from unbound ^{125}I-labeled monoclonal antibody;

g. detecting the amount of label bound to the immunoadsorbent; and

h. relating the amount of bound ^{125}I-label and the amount of preselected monoclonal antibody to determine the amount of preselected monoclonal antibody in the liquid sample.

17. An assay of Claim 16, wherein anti-idiotypic monoclonal antibodies are anti-idiotypic antibodies specific for the same private idiotope on the preselected monoclonal antibody.

18. An assay of Claim 16, wherein the preselected monoclonal antibody is HA-1A.

19. An assay kit for preselected monoclonal antibody in a biological sample from a human, including:

a. an immunoadsorbent containing anti-idiotypic monoclonal antibody specific for an idiotope of the preselected monoclonal antibody; and

b. labeled anti-idiotypic monoclonal antibody specific for an idiotope of the pre-selected monoclonal antibody.

20. An assay kit of Claim 19, wherein the anti-idiotypic monoclonal antibodies are anti-idiotypic antibodies specific for the same private idiotope of the preselected monoclonal antibody.

21. An assay kit of Claim 19 further including:

c. a preselected monoclonal antibody standard.

22. An assay kit for a preselected monoclonal antibody in a biological sample from a human including:

a. an immunoadsorbent comprising polystyrene beads with anti-idiotopic monoclonal antibody specific for an idiotope of the preselected monoclonal antibody affixed thereto;

b. ^{125}I-labeled anti-idiotypic monoclonal antibody specific for an idiotope of the preselected monoclonal antibody; and

c. preselected monoclonal antibody standard.

23. A kit of Claim 22, further comprising:

c. preselected monoclonal antibody standard.

24. A kit of Claim 22, wherein wherein the anti-idiotypic monoclonal antibodies are specific for the same private idiotope of the pre-selected antibody.

25. An assay of Claim 22, wherein the preselected monoclonal antibody is HA-1A.

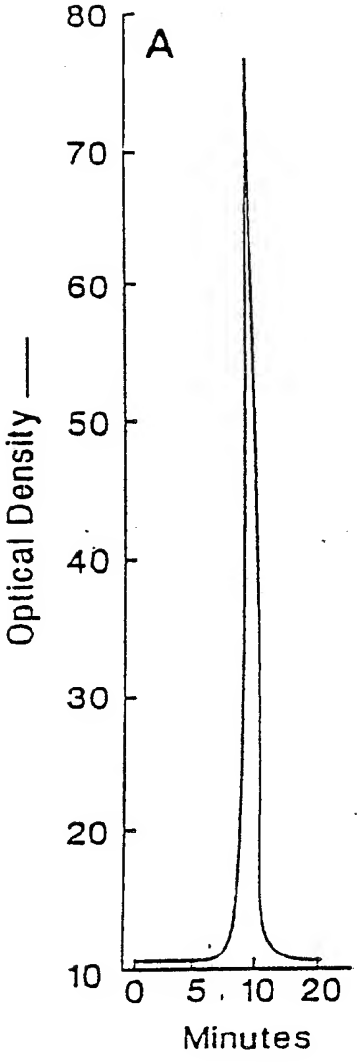


FIGURE 1A

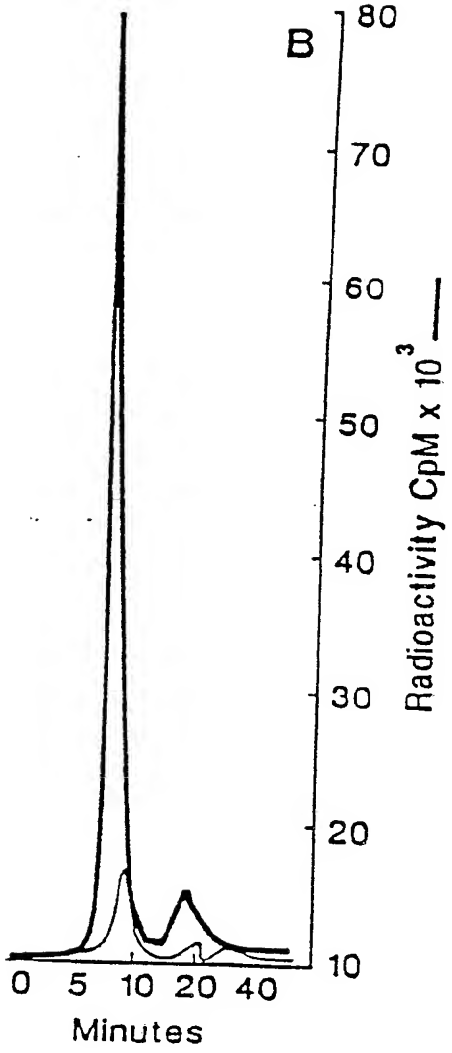
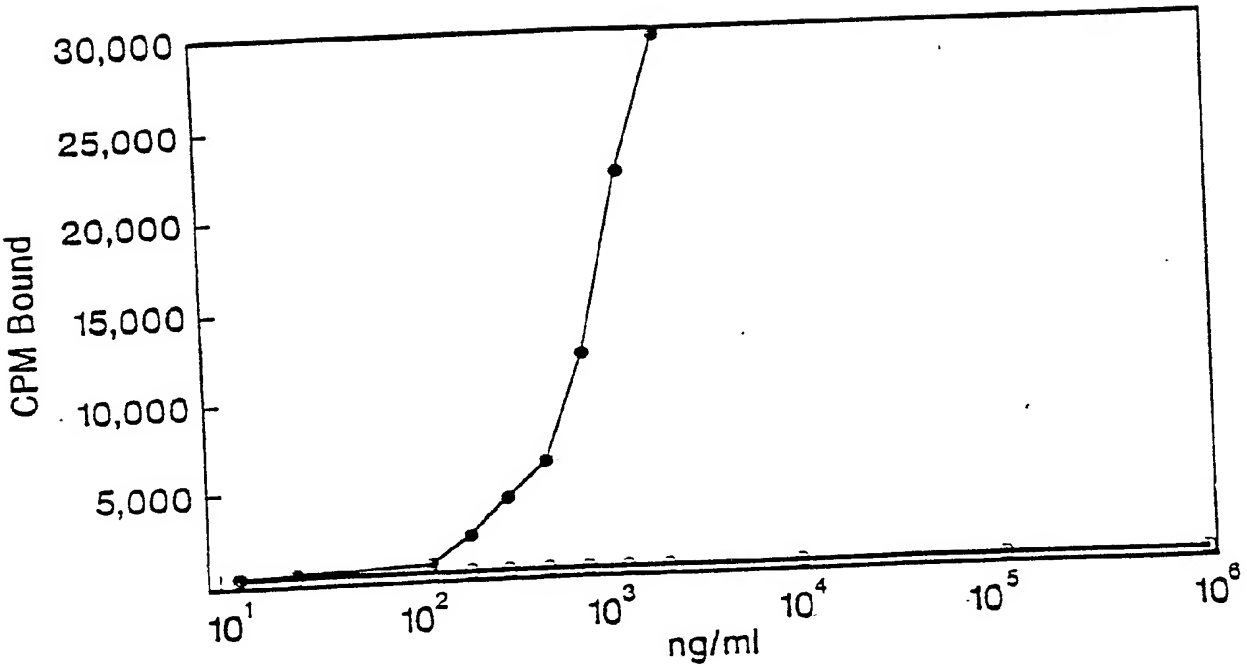


FIGURE 1B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CPM Bound
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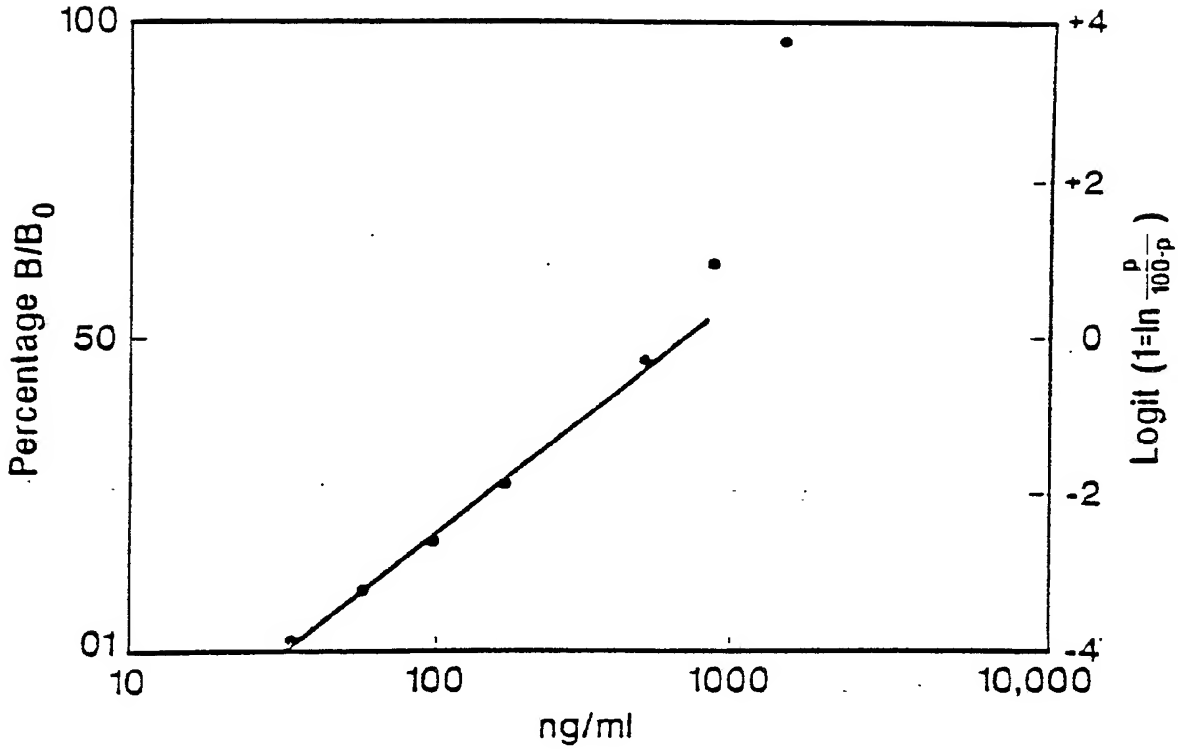
ng/ml

FIGURE 2


100
50
01
Percentage B/B0
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100
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ng/ml
+4
+2
0
-2
-4
Logit (1=ln p/100-p)

FIGURE 3

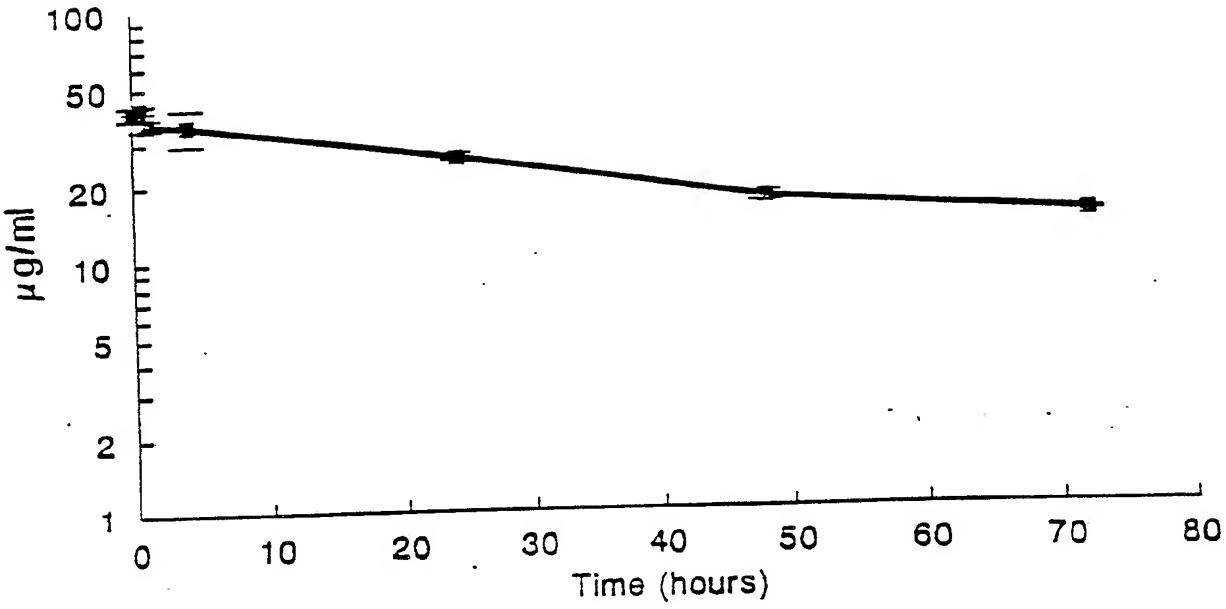

FIGURE 4

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 89/05569

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC : G 01 N 33/68, G 01 N 33/577

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC	G 01 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	WO, A, 88/06293 (D. LEWIS) 25 August 1988, see page 13, lines 11-14; page 14, lines 19-26; claims 2,5,8,17 --	1,3-7,9,10, 12-14,16, 17,19,20
A	EP, A, 0139389 (SYNBIOTICS CORPORATION) 2 May 1985, see page 9, lines 20-26; page 13, lines 11-26 --	1-25
A	EP, A, 0092249 (EISAI CO. LTD) 26 October 1983, see page 20, lines 1-10	2,6,12

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* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
5th April 1990	11. 05. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	F.W. HECK

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 8905569 SA 33574

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 04/05/90
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 8806293	25-08-88	EP-A- 0302109	08-02-89
EP-A- 0139389	02-05-85	US-A- 4828981	09-05-89
		JP-A- 60070361	22-04-85
EP-A- 0092249	26-10-83	JP-A- 58183629	26-10-83
		CA-A- 1194792	08-10-85
		DE-A- 3378301	01-12-88

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82